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Temperature Dependence of Mitochondrial Respiratory Activities

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Abstract

Arrhenius plots of succinate oxidase activity in intact beef heart mitochondria show a clear transition from a low to a high activation energy at 27° C. This temperature is significantly higher than that observed for ATPase (17° C). Arrhenius plots of succinate-cytochrome *c* reductase and cytochrome *c* oxidase also show anomalous curves; while the latter has a breakpoint (at 26° C) only when assayed manometrically, the former has a break at only 20° C.

The succinoxidase activity of lipid-deficient mitochondria depends upon addition of exogenous phospholipids. Unsaturated phospholipids are more active than saturated phospholipids but the latter become very effective in restoration of succinoxidase at increasing temperatures. It is suggested that a liquid-crystalline state of the phospholipids is required for correct binding to the lipid-depleted membrane and for restoration of respiratory activity. The is no clear correlation between the above mentioned effects in lipid deficient mitochondria and the transitions in the Arrhenius plots of intact mitochondria.

It is reported in the accompanying communication [1] that the mitochondrial ATPase activity presents a clear transition from low to high activation energy when the temperature decreases below 16-17° C. Such an effect, which has been observed by other investigators [2-4] has been related by us to the presence of phospholipids in a liquid-crystalline state [5] above this break point since solubilization of the membrane with Triton-X-100 and transformation of the ATPase into an oligomycin insensitive form decrease the gap in activation energies between the temperature regions above and those below the break.

While the ATPase is a protein complex which is partially "extrinsic" [6] to the inner mitochondrial membrane [cf. 7], the complexes of the electron transfer chain are tightly bound to the membrane [8] and the lipid requirement for their electron transfer activity is unanimously recognized [9, 10]; it has been shown that acetone extracts lipids from the inner mitochondrial membrane leaving an apparently intact morphological membranous appearance [11]; reintroduction of lipids into lipid-depleted membranes results in restoration of respiratory activity [9]. In view of the well known lipid dependence of mitochondrial respiration we have extended the study of the effect of temperature to respiratory activity in intact beef heart mitochondria; furthermore we have investigated the effect of different phospholipids at varying temperatures on the restoration of succinoxidase activity in lipid-deficient mitochondria.

Methods

Beef heart mitochondria (BHM) were prepared according to Smith [12]. BHM were extracted with 90% aqueous acetone according to Fleischer and Fleischer [13] in order to prepare lipid-deficient mitochondria (LDM). Phospholipids used in this study were the following: Asolectin (mixed soybean phospholipids from Associated Concentrates Inc , Woodside, New York; soybean lecithin, dimyristoyl lecithin, dioleyl lecithin, and dilinoleyl lecithin (from the Hormel Institute, Austin, Minn.); dipalmitoyl lecithin and dipalmitoyl phosphatidyl ethanolamine (from K and K Lab., Plainview, N.Y.); egg lecithin and egg phosphatidyl ethanolamine (from Koch-Light Lab., Canbrook, Bucks, England); myelin phospholipids (prepared from beef myelin according to ref. 14). The fatty acid composition of the phospholipid mixtures used, determined by means of gas-liquid chromatography is also reported in ref. 14. The different phospholipids were dispersed in 0.02 M Tris acetate buffer, pH 8, containing 1 mM EDTA and were sonicated [13]. Reconstitution from LDM and sonicated phospholipids was accomplished either during the preincubation in the manometric assays for succinoxidase or by incubation in a water bath at 30° for 30 min prior to assay at the desired temperature. The enzymic activities were determined according to the following procedure: succinoxidase was assayed manometrically [15]; succinate-cytochrome *c* reductase was determined spectrophotometrically [16] and cytochrome *c* oxidase either manometrically using ascorbate and N,N,N',N'-tetramethyl phenylendiamine (TMPD) as the substrate [17] or spectrophotometrically using an excess of reduced cytochrome *c* as the substrate [18]. Protein was measured with the method of Gornall *et al.* [19] and lipid phosphorus according to Marinetti [20].

Results

Arrhenius Plots of Enzymic Activities in Intact Mitochondria

Figure 1 shows an Arrhenius plot of succinoxidase activity in intact BHM. There is a clear break at 27° C, below which the activation energy is doubled. The activation energies calculated from the slopes in the Arrhenius plots are reported in Table I.

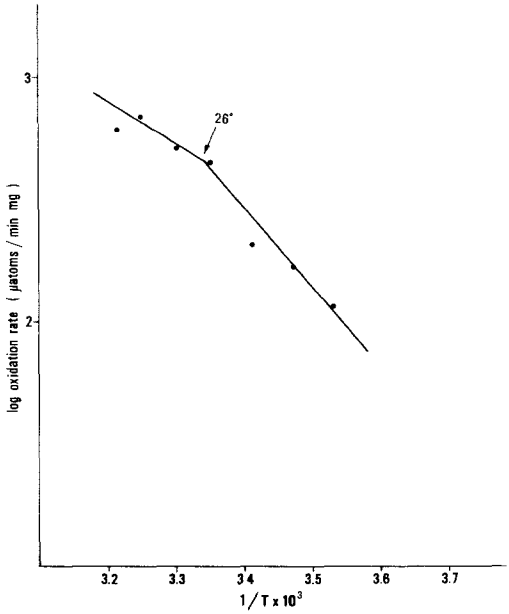

Figure 1. Arrhenius plot of succinate oxidase in BHM.

TABLE I. Break points and activation energies of mitochondrial respiratory activities

	Break temperature (°C)	Activation energies (kcal/mole) above transition temperature	below transition temperature
Succinate oxidase	27	9.1	17.0
Succinate-cytochrome *c* reductase	20	15.7	32.0
Cytochrome *c* oxidase[a]	—		9.3
Cytochrome *c* oxidase[b]	26	7.5	14.6

[a] Assayed spectrophotometrically with reduced cytochrome *c* as substrate.
[b] Assayed manometrically with ascorbate + TMPD as substrate.

The total oxidase activity from succinate may be considered the result of a stepwise transfer of electrons through three complexes of the respiratory chain [21]; we have studied the activation energies of the first two steps together (succinate to cytochrome *c* through complexes II and III of the chain [21]) and of the third step (reduced cytochrome *c* to oxygen, through Complex IV of the chain). Figure 2 shows the Arrhenius plot of succinate-cytochrome *c* reductase. There is a clear break in the slope as in the total oxidase, but the temperature at which the transition to a higher activation energy occurs is only 20°, in contrast with 27° of succinate oxidase.

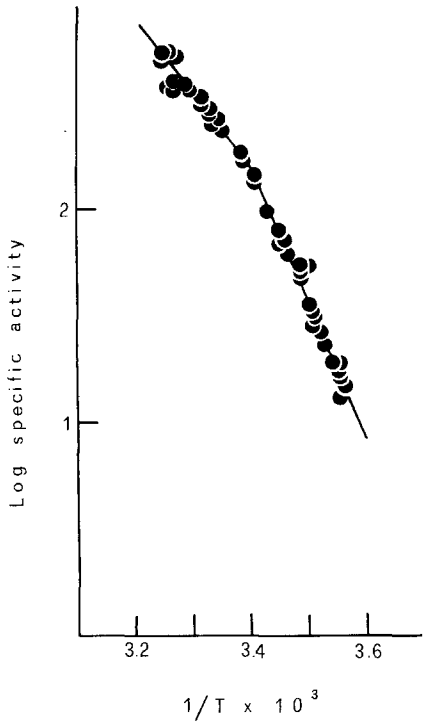

Figure 2. Arrhenius plot of succinate-cytochrome *c* reductase in BHM.

In Fig. 3 is reported an Arrhenius plot of cytochrome *c* oxidase. The enzyme assayed manometrically with ascorbate and TMPD shows a break at 26°, in good agreement with the value found for the total succinate oxidase (Fig. 3A); on the other hand the enzyme assayed spectrophotometrically with reduced cytochrome *c* as the substrate shows no break in the temperature range studied (Fig. 3B).

The activation energies for the partial activities are also reported in Table I.

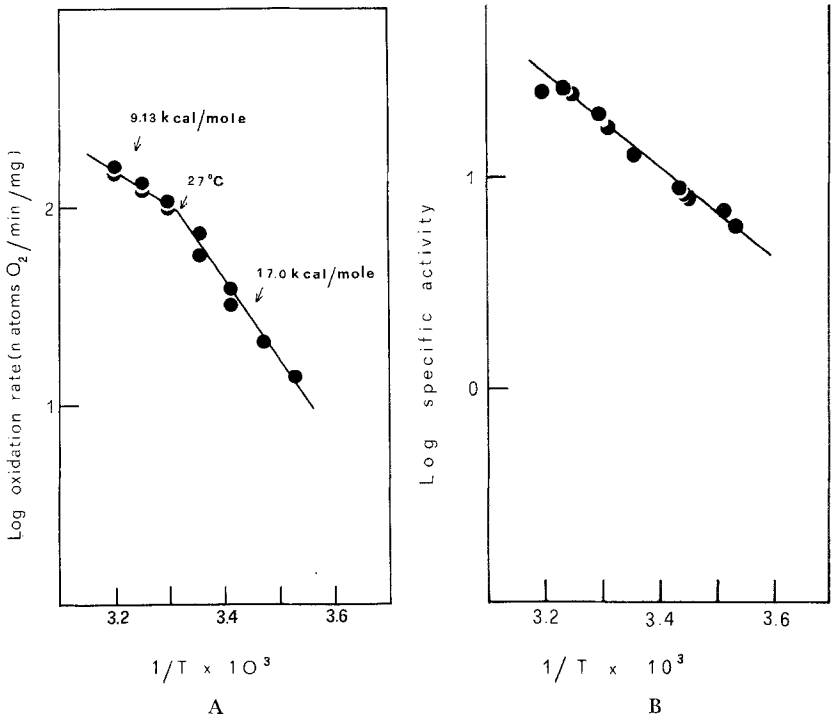

Figure 3. Arrhenius plot of cytochrome *c* oxidase in BHM. A. Assayed manometrically with ascorbate and TMPD as substrate. B. Assayed spectrophotometrically with reduced cytochrome *c* as substrate.

As shown in the accompanying paper [1], the mitochondrial ATPase activity has a clear breakpoint near 16-17°, which is lower in comparison with all of the respiratory activities examined here. Other mitochondrial activities which do not depend upon lipids, like malate dehydrogenase [22] or the nonenergy-linked pyridine nucleotide transhydrogenase [3] have no breaks through the same temperature ranges.

Studies in Lipid-deficient Mitochondria

The succinate oxidase activity of LDM is restored by adding mixed phospholipids [13]; we have previously observed that phospholipids having different fatty acid compositions do not reactivate succinoxidase to the same extent [14]. Table II shows that the respiratory activity of LDM at the standard assay temperature of 30° depends upon the nature of the added phospholipids: unsaturated phospholipids, like lecithin and phosphatidyl ethanolamine from egg are more active than saturated

TABLE II. Effect of different phospholipid fractions on restoration of succinoxidase activity and binding to LDM[a]

Phospholipid	Amount added (μg P/mg protein)	Phospholipid bound (μg P/mg)	Succinoxidase (μatoms O_2/min/mg)
Asolectin	54	17.3	0.48
Myelin PL[b]	53	17.2	0.32
PC (egg)	78	12.0	0.12
PC (dipalmitoyl)	54	41.6	0.00
PE (egg)	62	10.6	0.26
PE (dipalmitoyl)	50	15.5	0.05

[a] To 2 mg of LDM were added the appropriate phospholipids and incubated at 30° in the main compartment of the Warburg flasks. After 20 min the substrate was added from the side arm and the oxygen consumption recorded. At the end of the incubation period the contents of the flasks were centrifuged at 20,000 rpm for 10 min and the P content of the pellets was determined.

phospholipids (such as dipalmitoyl lecithin and dipalmitoyl phosphatidyl ethanolamine), which indeed are almost ineffective. The amounts of phospholipids bound are different for the different phospholipids but binding occurs in all cases.

However, when the effect of the same phospholipids is studied as a function of temperature (Fig. 4), it can be shown also saturated phospholipids gain activity in restoration of succinoxidase, at higher temperatures. Unsaturated phospholipids do not determine a progressive increase in activity as a function of temperature, but there is an optimum above which the activity decreases.

If the same curves are reported as Arrhenius plots (Fig. 5), no clear break temperatures can be determined. Figure 6 gives other examples of the effect of a number of commercial purified lecithins on the succinoxidase activity of LDM. Dimyristoyl lecithin becomes active only above 20°, while the unsaturated lecithins at first becomes progressively more active with increasing temperatures, then lose activity in restoration. Dilinoleyl lecithin is completely ineffective above 20°.

Discussion

The presence of breaks in the Arrhenius plots of respiratory activities is in accord with the findings of Lyons and Raison [23-25] for omeothermic animals and chilling-sensitive plants, in contrast with poichilothermic animals and chilling-resistant plants. What is unexpected is the finding that in our experiments there is a significantly different break temperature between succinate cytochrome *c* reductase and

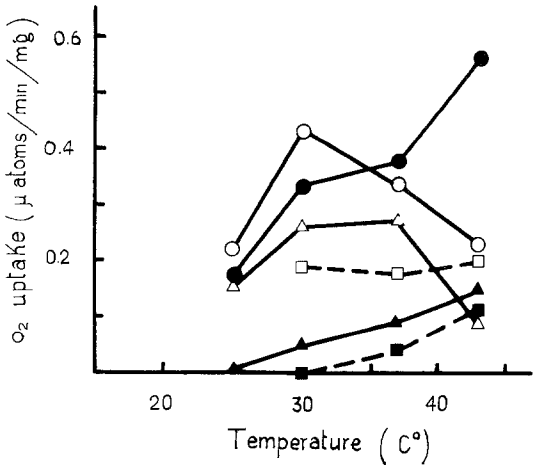

Figure 4. Effect of temperature on succinoxidase activity of LDM reconstituted with different phospholipids. ○——○, Asolectin; ●——●, myelin phospholipids; △——△, egg phosphatidyl ethanolamine; □——□, egg lecithin; ▲——▲, dipalmitoyl phosphatidyl ethanolamine; ■——■, dipalmitoyl lecithin.

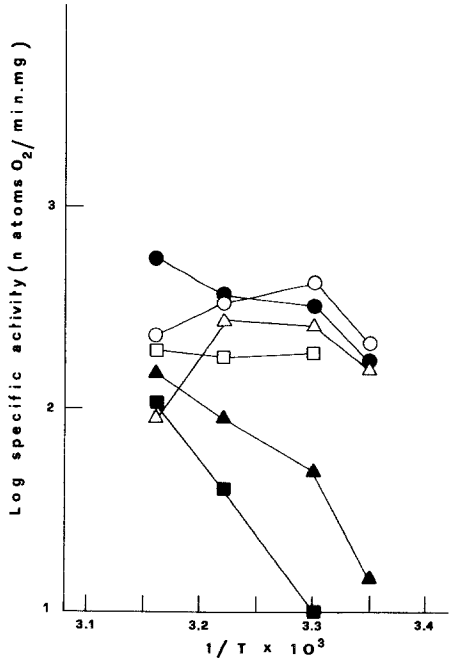

Figure 5. Arrhenius plots of succinoxidase in LDM reconstituted with different phospholipids. Symbols are the same as in the legend of Figure 4.

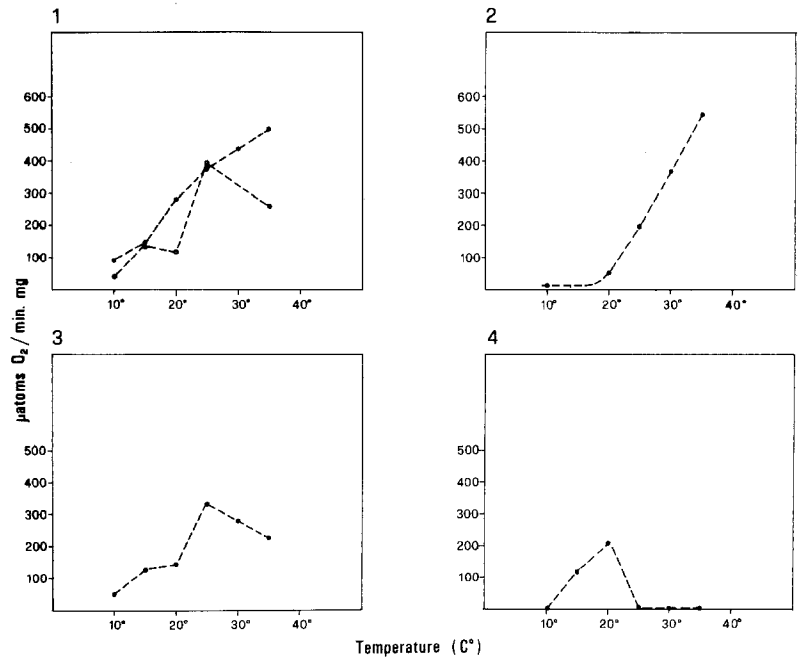

Figure 6. Effect of temperature on succinoxidase activity of LDM reconstituted with different lecithins. 1. Asolectin as a control (●——●) and soybean lecithin (○——○). 2. Dimyristoyl lecithin. 3. Dioleyl lecithin. 4. Dilinoleyl lecithin.

cytochrome oxidase, which in turn has about the same break as the total succinate oxidase. The reason why no break was observed when cytochrome oxidase was assayed with reduced cytochrome *c* as the substrate is not clear; however, the excess of cytochrome *c*, which is a basic protein and ionically binds to lipid bilayers [10, 26] may have modified the lipid fluidity of the membrane: an immobilizing effect of cytochrome *c* on phospholipid vesicles has been recently reported [27].

Anyway, all of the break temperatures for respiratory activities differ from that of the mitochondrial ATPase, which is lower, as shown in the accompanying paper [1]. The breaks observed by Raison *et al.* [24] for respiration in rat liver mitochondria coincided with the transition temperatures of the mitochondrial phospholipids studied by the motion of spin-labelled fatty acids. On the other hand Blazyk and Steim [28], also studying rat liver mitochondria, have produced evidence by means of differential scanning calorimetry that the transition temperatures of the phospholipids both isolated and in the membranes are centered near 0°, although spanning a large temperature range. The differences observed may be due to the techniques employed: it was suggested [29]

that spin labels preferentially are localized in the more fluid regions of phospholipid bilayers. Moreover, since the transition temperature of the phospholipids is related to their unsaturation [30], and the unsaturation may change with the lipid composition of the diet [31], may differences may also depend upon changed experimental conditions. It is also plausible that beef heart mitochondria (which have been used in this study) have higher transition temperatures of their phospholipids in comparison with rat liver mitochondria, since it is well known that lipids in ruminants have lower unsaturation than in monogastric animals [32]. As a mere indicative comparison, in Table III we report the fatty acid composition of beef heart mitochondrial phospholipids determined in our laboratory and the fatty acid composition of rat liver mitochondria also from our laboratory. (Unfortunately, in the studies cited above the fatty acid composition of rat liver mitochondria was not reported).

TABLE III. Fatty acid composition of beef heart and rat liver mitochondrial phospholipids[a].

Fatty acid	Beef heart	Rat liver
14.0	2.80[b]	0.21
16.0 ald.	5.00	—
16.0	9.94	20.57
16.1	4.12	2.96
18.0	18.81	18.41
18.2	26.40	19.71
20.4	9.69	14.10
20.5	1.48	0.87
22.6	traces	9.20

[a] Determined by gas liquid chromatography using a diethylene glycol succinate column as previously described [14].

[b] Fatty acids are reported as per cent of total fatty acids eluted from the gas-chromatographic column. Only major acids are shown.

Since the interpretation of the Arrhenius plots is not univocal [1, 2], we have addressed ourselves to study the respiratory activity of lipid-deficient mitochondria extracted with aqueous acetone (LDM): it has been found that any phospholipid is active in restoration of respiration, provided that it be assayed at an appropriate temperature, conceivably above the transition temperature. For example, dimyristoyl lecithin has little effect at 20°, when dioleyl lecithin is very effective, but becomes much more active than dioleyl lecithin above 30°. On the other hand dipalmitoyl lecithin is active only above 37°.

An unexplained effect in this experiment was the decrease in respiratory activity of mitochondria reconstituted with unsaturated fatty acids; for example dilinoleyl lecithin has little activity above 20°.

Perhaps an optimal temperature is required for each type of lipids either for respiratory activity or for a correct interaction with mitochondrial proteins. In other words phospholipid binding to LDM appears to require an optimum fluidity of the fatty acyl chains. However, the decrease in activity of unsaturated phospholipids at higher temperatures might also reflect an increased rate of denaturation. The reason why denaturation may be increased with unsaturated lipids and not with saturated lipids may reflect membrane organization or an effect of increased lipid peroxidation and requires further studies. The important fact, in our opinion, is the demonstration that phospholipids having any kind of fatty acid composition are active provided that they be assayed at an appropriate temperature. The fact that phospholipids are essentially ineffective below the transition temperatures supports the view expressed in the accompanying communication [1] that breaks in the Arrhenius plots of membrane activities may be related to the lipid fluidity but not to the main phase changes shown calorimetrically. The possibility that different enzymatic proteins in membrane are contained in a specific microenvironment having a well defined degree of fluidity deserves serious consideration.

Acknowledgements

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